Fabrication of micro-alginate beads under centrifugally induced artificial gravity conditions

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National Institute of Technology, Rourkela

Certificate

This is to certify that the report entitled "**Fabrication of micro-alginate beads under centrifugally induced artificial gravity conditions**" submitted by **Mr. RAHUL KUMAR**, Roll No.: **111BT0548**, B-Tech-8th semester, Department of Biotechnology & Medical Engineering, National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the report has not been submitted to any other University / Institute for the award of any Degree or Diploma.

Date: 26 th June 2015

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RAHUL KUMAR

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ABSTRACT

This work presents a method for the direct, centrifugally induced fabrication of small, Ca^{2+} cross-linked alginate beads at syringe needle micro-nozzles. The bead diameter was found between 65-282.5 μm and rpm between 1900-200 rpm. Centrifuge tube-syringe set up is aligned vertically at rest in flying bucket and under rotation they align horizontally. The centrifugally induced, ultra-high artificial gravity conditions allow the micro-encapsulation of alginate solutions. With this low cost technology for fabrication of micro alginate beads, beads with less than 300 μm have been formed.

Keywords: Centrifugal, syringe, droplet formation, alginate micro-bead

LIST OF FIGURES

Figure 16: Variation of minimum rpm at which beads formed with respect to its alginate concentration for (a) 26G needle and (b) 30G needle.

CHAPTER-1
INTRODUCTION

1.1 INTRODUCTION

Microencapsulation is a process in which small particles are surrounded by a coating to give protection. Three major areas where microencapsulation is in use are-food production, cosmetic industry, drug delivery [1].

In the area of food production, enzymes and vitamins are encapsulated to increase the effect of nutrition. Similarly for cosmetic industry, colours and flavours are encapsulated [2]. Conventional techniques like spray drying is used for these encapsulation as diameter of these encapsulated beads requires 100-1000 μm [3]. Drug delivery is another field where two things are focussed. One is encapsulation and other is release of pharmaceuticals in controlled manner, e.g. for cancer therapy. The living cells are encapsulated to replace the failed body functions, e.g. in diabetes. This requires small size encapsulation for diffusive mass transport [4].

This project focuses on formation of micro-alginate beads and the encapsulation of living cells into biocompatible and biodegradable polymer like alginate which are being investigated for *in-vivo* application. Biopolymer like alginate has been extensively used here. Alginate is an anionic polysaccharide or biopolymer which is found in cell wall of brown algae. Sodium alginate solution hardens once it comes in contact with Ca^{2+} ions [5]. The coatings of calcium alginate is semi-permeable in nature. The immune system do not recognize the encapsulated alien cells. The cells remain in healthy conditions due to available of nutrition thorough diffusive transport of nutrition and allowing metabolic process to continue. Thus it is used in implantation. For cell therapy, the optimum size 50-300 μm has been suggested. In 1908, the insulin producing pancreatic cells were encapsulated into alginate beads [6].

Production of micro-alginate beads require two challenges to be overcome. First the surface tension and viscous force which stops the droplet to break-off at the needle tip must overcome. Secondly, there should not be clogging or agglomeration. There are two major ways for cell encapsulation [7].

Direct method implies the passage of alginate solution through air gap into $CaCl_2$ solution where they are instantaneously hardened [8]. The air gap prevents premature hardening and clogging at the nozzle. The vibration at the nozzle causes droplet to break-off. But these technique requires complex and costly apparatus.

Indirect methods requires removal of oil from the surface of bead thus making throughput of these indirect method less than that of direct method [9].

This project uses the technique of centrifugally induced micro-encapsulation. This novel technique can form microbeads with highly viscous alginate solution without compromising on the vitality of cells. Here a centrifuge tube-syringe setup is made where alginate solution contained in syringe is diffusion based hardened by $CaCl_2$ solution present in centrifuge tube.

CHAPTER –2

LITERATURE REVIEW

2.1 LITERATURE REVIEW:

2.1.1.1 Sodium alginate

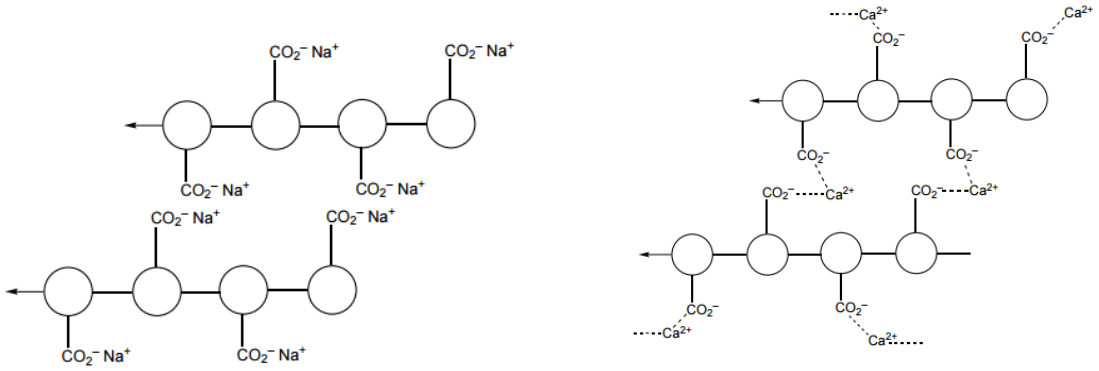
It is cationic polysaccharide found in cell wall of brown algae. It is biocompatible and is used as biomaterial. The random sequence of Mannuronic acid(M) and Guluronic acid(G) comprises alginate. It's empirical formula is $NaC_6H_7O_6$. It is used in food industry, removing radioactive substance from body since it is an effective chelator, immobilisation of cells to obtain alcohols [10].

2.1.1.2 Calcium alginate

It is prepared by adding $CaCl_2$ solution with aqueous sodium alginate. It's chemical formula is $C_{12}H_{14}CaO_{12}$. It is widely used in medicine industry. It includes burn dressings which helps to heal. It is widely used in immobilisation and encapsulation due to it's biocompatibility and gelation property [11].

2.1.2. Crosslinking mechanism

Alginate polymer can be shown as in fig. 1(a).



(a) (b)

Fig. 1: Crosslinking mechanism

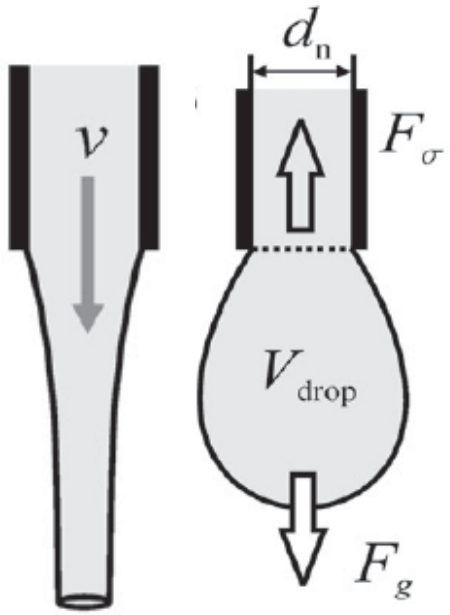
Calcium ions is used for cross linking because of its non-toxicity. When calcium ions in the solution come in contact with sodium alginate, the calcium ions replace the sodium ions in the polymer [12]. Each calcium ions attach to two of the polymer strands. This is called cross linking and in represented in fig. 1(b).

2.1.3 Bead formation:

Alginate forms gels with wide range of cations. Size of beads can be controlled by regulating various parameters. As we increase the concentration of calcium ions, the positive calcium ions take up places next to the negative ions and there lies less space for water molecules. This makes hydrogel beads loose some water. The negative charges along the chain repel each other less in the presence of sodium ions and so chains become more coiled up and this squeezes out water from bead [13].

2.1.4 Principle of operation:

There are two basic droplet formation mechanism for a nozzle of inner diameter d_n exposed to gravity which is depicted in fig. 2.



(a) (b)

Fig. 2: Droplet formation mechanism (adapted from [7])

As gravitational force Fg exceeds the surface tension induced counter force, there is droplet formation as depicted in fig. 2(a). A jet is issued out of nozzle at high flow rate. As depicted in fig. 2(b). By equating the two forces, we can calculate the theoretical bead diameter d_{drop} where d_n in diameter of nozzle, σ_{drop} is surface tension of drop, ρ in density of drop [14].

$$d_{drop} = \sqrt[3]{\frac{6\, d_n\, \sigma_{drop}}{\rho_{drop}\, g}}$$

But by using centrifugation, one can produce artificial gravity conditions and g can be expressed as $\omega^2 r$ where ω is angular frequency and r is radial position of nozzle.

2.1.5 Encapsulation

Microencapsulation is a process in which small particles are surrounded by a coating to give protection. Three major areas where microencapsulation is in use are-food production, cosmetic industry, drug delivery [15].

2.1.5.1 Reasons for microencapsulation

It is used to prolong the life of products that are encapsulated, control its liberation in appropriate time and space. The product need to be isolated from the surrounding, like in case of vitamins isolated to prevent it from harmful effects of oxygen, reducing evaporation of volatile core, separating reactive core from chemical attack [16]. The objective is not to isolate core from surrounding but to control the rate at which it leaves and reaches to its surrounding as in controlled release of drug or pesticides. Encapsulation of cells is of important concern here [17]. The immune system do not recognize the encapsulated alien cells. The cells remain in healthy conditions due to available of nutrition thorough diffusive transport of nutrition and allowing metabolic process to continue [18]. Thus it is used in implantation. Here the strength and elasticity of microcapsule must remain high for long time and smooth surfaces are required to prevent immunologic reactions [19].

2.1.5.2 Cell encapsulation:

It involves immobilisation of cells within polymer that allows bidirectional diffusion of molecules such as growth factors, influx of oxygen, nutrients which are necessary for cell metabolism and diffusion of waste products and therapeutic proteins [20]. It also prevents cells from immune cells and antibodies. The idea behind cell encapsulation is to overcome the problem of graft rejection in tissue engineering. Though therapeutic products can be injected at the site of implantation , encapsulated cells would provide therapeutic products to the affected site in a controlled and for longer duration [21]. In drug delivery implantation of drug loaded artificial cell would be more cost effective that direct drug delivery and prolonged drug deliver [22].

It is necessary to ensure that the microcapsule has adequate mechanical stability to withstand physical and osmotic stress such as during the exchange of nutrients and waste products. [23]The microcapsules should be strong enough and should not rupture on implantation as this could lead to an immune rejection of the encapsulated cells. [24]For instance, in the case of xenotransplantation, a tighter more stable membrane would be required in comparison to allo-transplantation [25].

2.1.5.2.1 Non-Therapeutic applications

Cell microencapsulation is used in food industry for the encapsulation of live probiotic bacteria cells. This is done to prolong bacteria viability during processing of diary products and targeted delivery to the gastrointestinal tract [26].

2.1.5.2.2 Therapeutic Applications

For diabetes microencapsulation technique can make use of animal cells or genetically modified insulin producing cells or protect the islets cells from immune response. These islets encapsulated microcapsules could prevent the need for insulin injections [27].

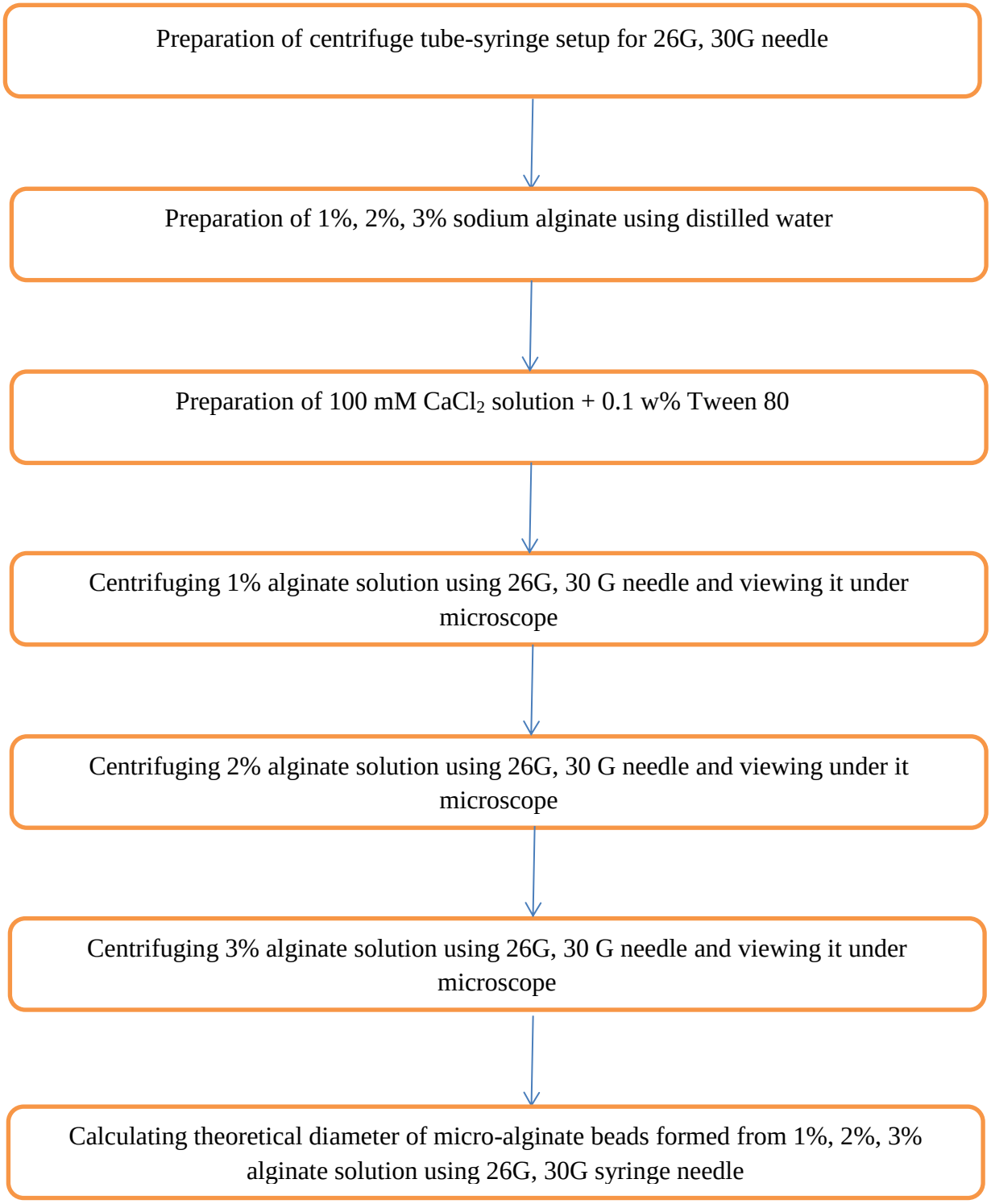
For cancer, there can be cure by implantation of microcapsules containing genetically modified cytokine secreting cells [28]. The effect of implanting microcapsules loaded with xenogenic cells genetically modified to secrete endostatin, an antiangiogenic drug which causes apoptosisin tumor cells, has been extensively studied [29].

CHAPTER – 3
OBJECTIVES AND WORK PLAN

3.1Objectives

- Finding the minimum rpm at which micro-alginate beads formed.
- Preparation of micro-beads between 50-300 µm which is good for implantation.
- Studying the effect of micro-alginate bead diameter with rpm and concentration.

3.2 Work Plan

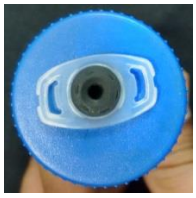

Preparation of centrifuge tube-syringe setup for 26G, 30G needle
Preparation of 1%, 2%, 3% sodium alginate using distilled water
Preparation of 100 mM $CaCl_2$ solution + 0.1 w% Tween 80
Centrifuging 1% alginate solution using 26G, 30 G needle and viewing it under microscope
Centrifuging 2% alginate solution using 26G, 30 G needle and viewing under it microscope
Centrifuging 3% alginate solution using 26G, 30 G needle and viewing it under microscope
Calculating theoretical diameter of micro-alginate beads formed from 1%, 2%, 3% alginate solution using 26G, 30G syringe needle

CHAPTER – 4
MATERIALS AND METHODS

4.1 Preparation of centrifuge tube-syringe setup

4.1.1 For 26G needle

Using driller machine, holes were made on centrifuge cap as shown in fig.3(a). Then holes were made on both sides of 2 ml syringe as shown in fig. 3(b). This was done for 10 number of 2ml syringe as shown in fig.3(c). Then centrifuge caps were holed and syringe was inserted into hole made in centrifuge cap as shown in fig. 3(d). 50 ml centrifuge tube were holed on both sides. This was done for 10 number of centrifuge tube. Then the centrifuge cap was inserted into centrifuge tube to make the complete setup as shown in fig. 3(e).



(a)

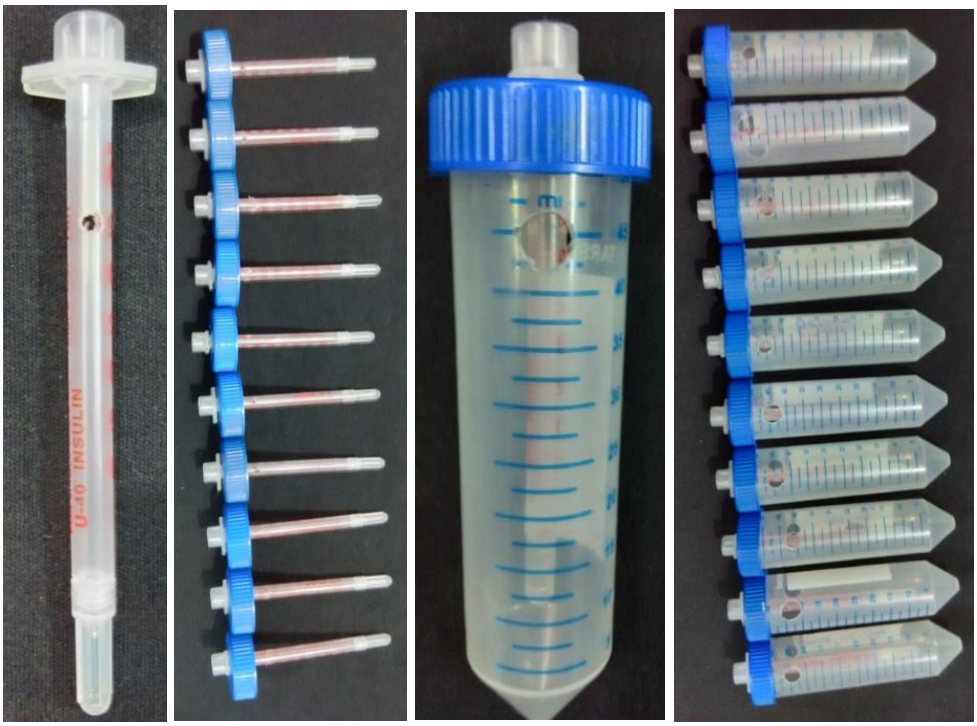
(b) (c) (d) (e)

Fig. 3: Centrifuge tube-syringe setup for 26G syringe made by making holes in (a) centrifuge cap (b) syringe (c) 10 centrifuge cap and syringe (d) centrifuge tube (e) 10 centrifuge tube.

4.1.2 For 30G needle

Using driller machine, holes were made on centrifuge cap as shown in fig.4(a). Then holes were made on both sides of 1 ml syringe as shown in fig. 4(b). This was done for 10 number of 1ml syringe. Then centrifuge caps were holed and syringe was inserted into hole made in centrifuge caps. 10 centrifuge caps were holed as shown in fig. 4(c). 50 ml centrifuge tube were holed on both sides as shown in fig. 4(d). This was done for 10 number of centrifuge tube. Then the centrifuge cap was inserted into centrifuge tube to make the complete setup as shown in fig. 4(e).

(a)



(b) (c) (d) (e)

Fig. 4: Centrifuge tube-syringe setup for 30G syringe made by making holes in (a) centrifuge cap (b) syringe (c) 10 centrifuge cap and syringe (d) centrifuge tube (e) 10 centrifuge tube.

4.2 Preparation of 1%, 2%, 3% alginate solution

4.2.1 For preparing 1% alginate solution.

99 ml of distilled water was taken in 500 ml beaker and kept on magnetic stirrer at 400 rpm and 1 g of alginate was poured in distilled water slowly and kept overnight for 16 hours.

4.2.2 For preparing 2% alginate solution.

98 ml of distilled water was taken in 500 ml beaker and kept on magnetic stirrer at 500 rpm and 2 g of alginate was poured in distilled water slowly and kept overnight for 16 hours.

4.2.3 For preparing 3% alginate solution

97 ml of distilled water was taken in 500 ml beaker and kept on magnetic stirrer at 600 rpm and 3 g of alginate was poured in distilled water slowly and kept overnight for 16 hours.

4.3 Preparation of 100 mM $CaCl_2$ solution with 0.1 w% Tween-80

For preparing 100 ml of 100 mM $CaCl_2$, 1.109 g of $CaCl_2$ was dissolved in measuring cylinder containing distilled water till volume of solution becomes 100 ml. 100 µl of tween-80 was dissolved in prepared solution.

4.4 Experimental setup

In this novel, rotor based set-up, droplets were generated within the dripping regime at the micro-nozzle tip of a commercially available syringe. The syringe contains alginate solution and centrifuge tube contains $CaCl_2$ solution. Therefore, the alginate droplet impacts perpendicular to the air–liquid meniscus within the tube. Upon halting the rotor, gravity prevails to realign the tube in a vertical position. The Centrifuge tube-syringe setup can be taken out of the rotor for further processing, e.g. culturing or analysis. Due to the intrinsic rotational symmetry, several nozzles can be operated simultaneously on the same rotor.

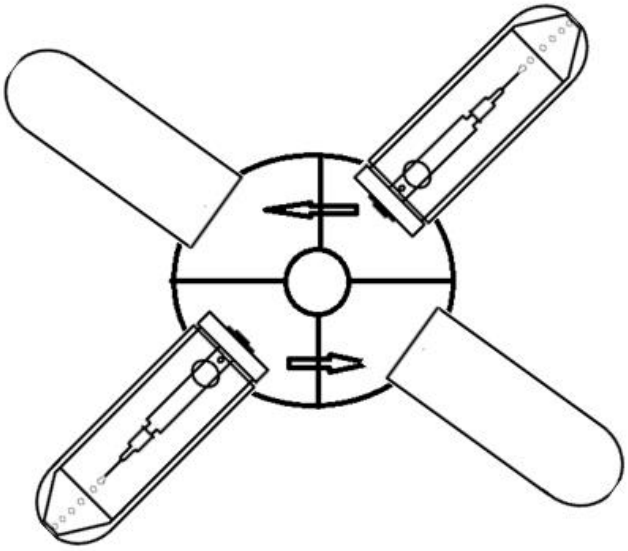


Fig.5: Schematic of the experimental setup consisting of centrifugal platform and centrifuge tube-syringe setup in a swinging bucket

4.5 Centrifugation and viewing it under microscope

The centrifuge tube-syringe setup was put inside centrifuge machine and cap was put on and centrifuge lid was closed and temperature, time, rpm was set. The formed beads were viewed under microscope.

(a) **(b)**

(c)

Fig. 6: (a) Centrifuge tube-syringe set up was kept in centrifuge machine (b) the cap was put on (c) rpm, temperature, time was set and centrifuge was run.

4.5.1 Formation of beads for 1% alginate solution using 26G needle.

Ten setup was marked as A, B, C, D, E, F, G, H, I, J. A and B were used as test to find minimum rpm at which beads formed. 5 ml of $CaCl_2$ was put in each centrifuge tube and 1 ml of alginate solution in each syringe.

4.5.2 Formation of beads for 1% alginate solution using 30G needle.

Ten setup was marked as A, B, C, D, E, F, G, H, I, J. A and B were used as test to find minimum rpm at which beads formed. 5 ml of $CaCl_2$ was put in each centrifuge tube and 0.25 ml of alginate solution in each syringe.

4.5.3 Formation of beads for 2% alginate solution using 26G needle.

Ten setup was marked as A, B, C, D, E, F, G, H, I, J. A and B were used as test to find minimum rpm at which beads formed. 5 ml of $CaCl_2$ was put in each centrifuge tube and 1 ml of alginate solution in each syringe.

4.5.4 Formation of beads for 2% alginate solution using 30G needle.

Ten setup was marked as A, B, C, D, E, F, G, H, I, J. A and B were used as test to find minimum rpm at which beads formed. 5 ml of $CaCl_2$ was put in each centrifuge tube and 0.25 ml of alginate solution in each syringe.

4.5.5 Formation of beads for 3% alginate solution using 26G needle.

Ten setup was marked as A, B, C, D, E, F, G, H, I, J. A and B were used as test to find minimum rpm at which beads formed. 5 ml of $CaCl_2$ was put in each centrifuge tube and 1 ml of alginate solution in each syringe.

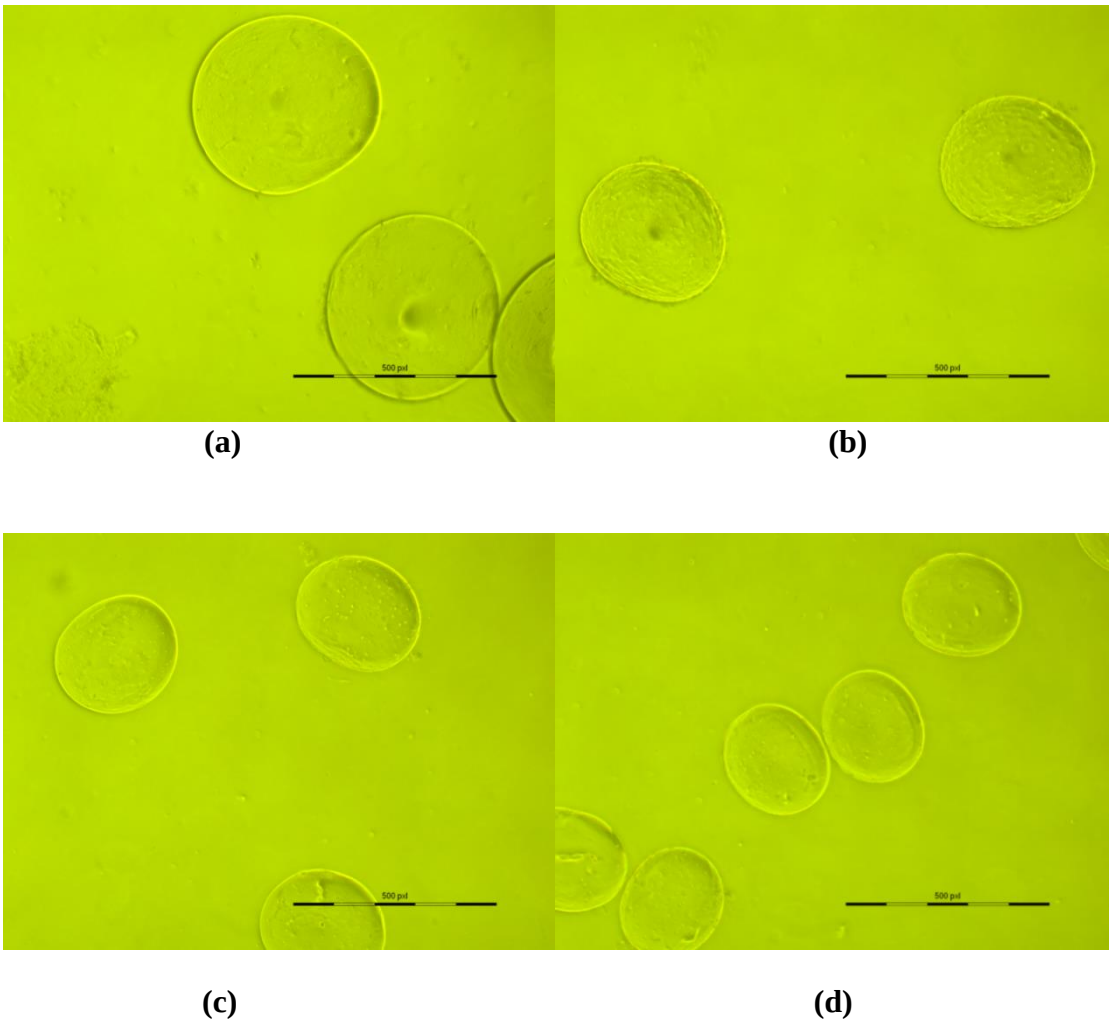
4.5.6 Formation of beads for 3% alginate solution using 30G needle.

Ten setup was marked as A, B, C, D, E, F, G, H, I, J. A and B were used as test to find minimum rpm at which beads formed. 5 ml of $CaCl_2$ was put in each centrifuge tube and 0.25 ml of alginate solution in each syringe.

CHAPTER - 5
RESULTS AND DISCUSSION

5.1 Micro-alginate beads from 1% alginate solution with 26G Syringe (O.D (450 µm), I.D (250 µm)):

300 rpm was the least rpm at which beads formed. Diameter of beads was found to be 282.5 µm as shown in fig. 7(a). The formation of beads was checked for three other rpm, say 400, 500, 600 rpm and the diameter of microbeads was found as 227.5 µm as shown in fig. 7(b), 185 µm as shown in fig. 7(c), 172.5 µm as shown in in fig. 7(d) respectively. From the graph it can be seen that as the rpm increased the bead diameter decreased.



(a) **(b)**

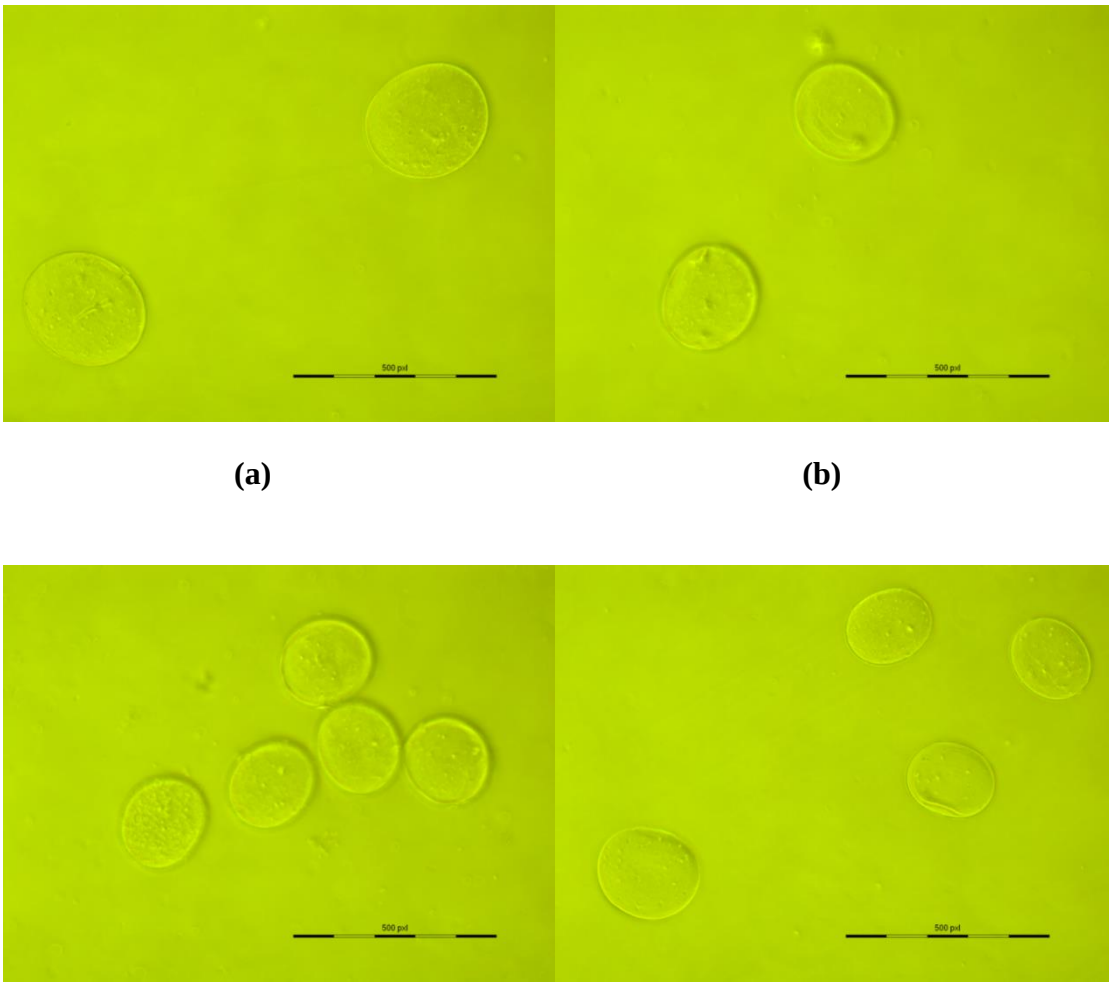
(c) **(d)**

Fig. 7: Micro-alginate beads formed at (a) 400 rpm having diameter 282.5 µm (b) 500 rpm having diameter 227.5 µm (c) 600 rpm having diameter 185 µm (d) 700 rpm having diameter 172.5 µm using 1% alginate and 26G syringe.

5.2 Micro-alginate beads from 1% alginate solution with 30G syringe needle (O.D (300 µm), I.D (148 µm))

There was no formation of beads at 400 rpm. 500 rpm was the least rpm at which beads formed. Diameter of beads was found to be 190µm as shown in fig. 8(a). The formation of beads was checked for three other rpm, say 600, 700, 800 rpm and the diameter of

microbeads was found as 162.5 µm, 157.5 µm, 132.5 µm. From the graph it can be seen that as the rpm increased the bead diameter decreased.



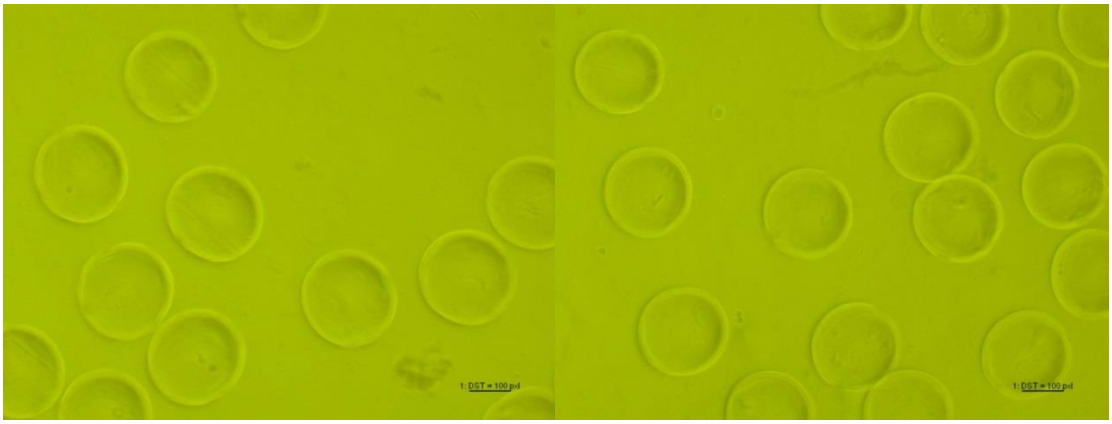
(a) **(b)**

(c) **(d)**

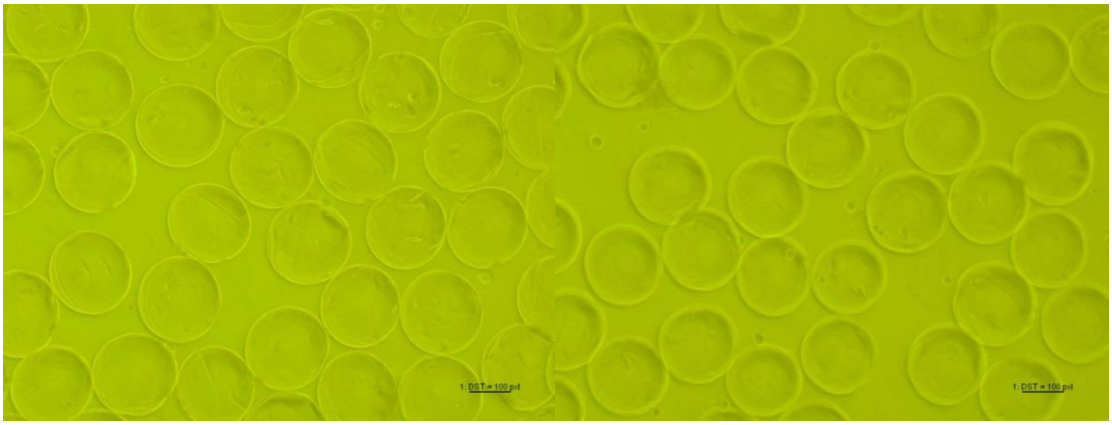
Fig.8: Micro-alginate beads formed at (a) 500 rpm having diameter 190 µm (b) 600 rpm having diameter 162.5 µm (c) 700 rpm having diameter 157.5 µm (d) 800 rpm having diameter 132.5 µm using 1% alginate and 30G syringe.

5.3 Micro-alginate beads from 2% alginate solution with 26G syringe needle (O.D (450 µm), I.D (250 µm)):

There was no formation of beads at 800 rpm. 900 rpm was the least rpm at which beads formed. Diameter of beads was found to be 145 µm as shown in fig. 9(a). The formation of beads was checked for three other rpm, say 600, 700, 800 rpm and the diameter of microbeads was found as 140 µm as shown in fig. 9(b), 135 µm as shown in fig. 9(c), 130 µm as shown in fig. 9(d). From the graph it can be seen that as the rpm increased the bead diameter decreased.



(a) **(b)**

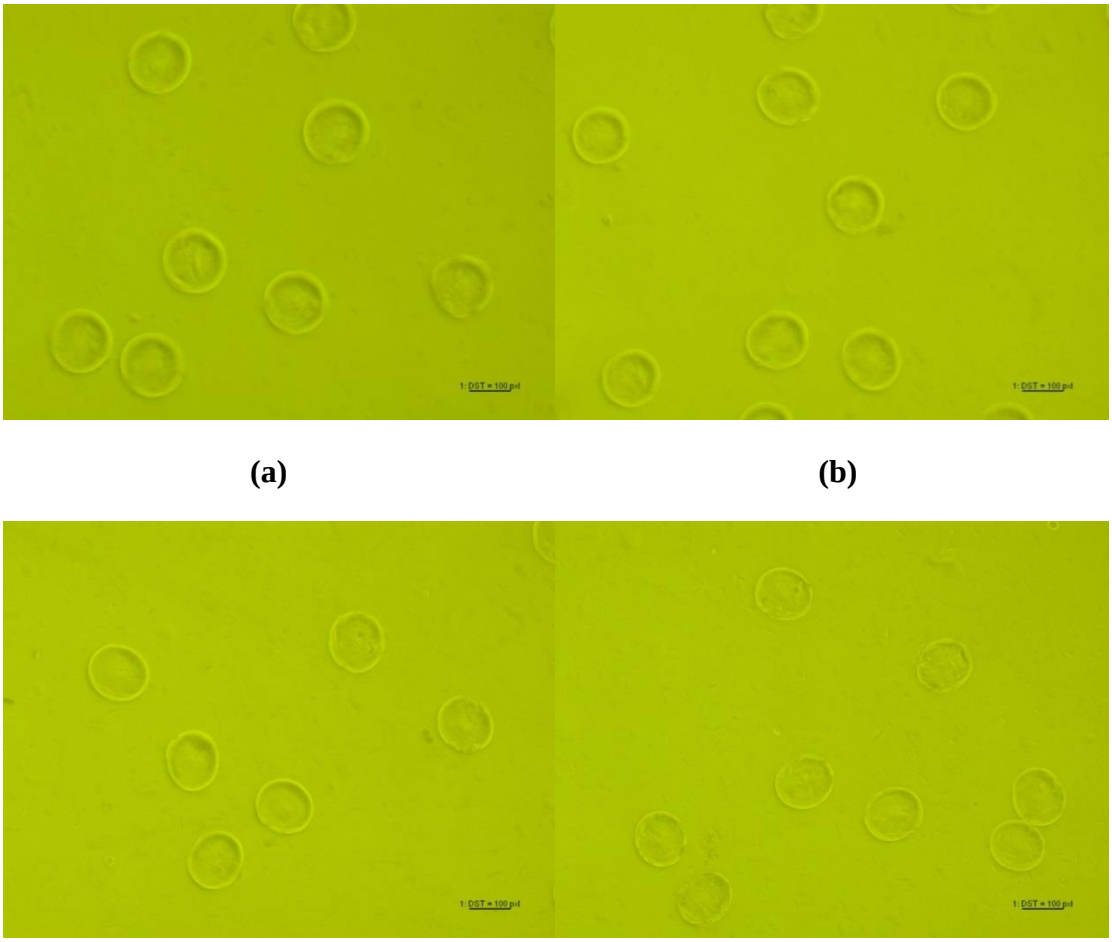


(c) **(d)**

Fig. 9: Micro-alginate beads formed at (a) 900 rpm having diameter 145 µm (b) 1000 rpm having diameter 140 µm (c) 1100 rpm having diameter 135 µm (d) 1200 rpm having diameter 130 µm using 2% alginate and 26G syringe.

5.4 Micro-alginate beads from 2% alginate solution with 30G syringe needle (O.D (300 µm), I.D (148 µm))

There was no formation of beads at 1400 rpm. 1500 rpm was the least rpm at which beads formed. Diameter of beads was found to be 110 µm as shown in fig. 10(a). The formation of beads was checked for three other rpm, say 1600, 1700, 1800 rpm and the diameter of microbeads was found as 95 µm as shown in fig. 10(b), 90 µm as shown in fig. 10(c), 87.5 µm as shown in fig. 10(d). From the graph it can be seen that as the rpm increased the bead diameter decreased.



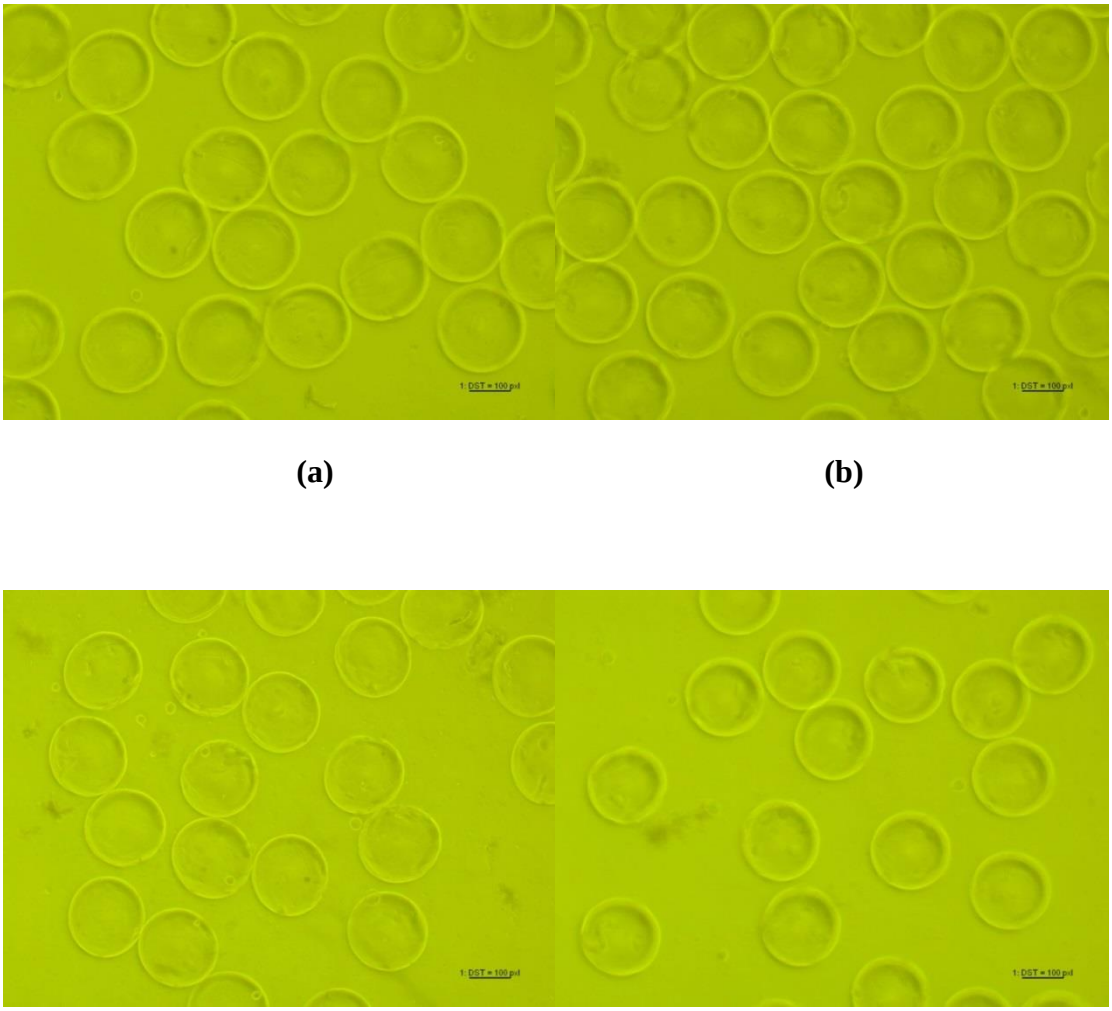
(a) **(b)**

(c) **(d)**

Fig. 10:Micro-alginate beads formed at (a) 1500 rpm having diameter 110µm (b) 1600 rpm having diameter 95 µm (c) 1700 rpm having diameter 90 µm (d) 1800 rpm having diameter 87.5 µm using 2% alginate and 30G syringe.

5.5 Micro-alginate beads from 3% alginate solution with 26G syringe needle (O.D (450 µm), I.D (250 µm)):

There was no formation of beads at 1000 rpm. 1100 rpm was the least rpm at which beads formed. Diameter of beads was found to be 145 µm as shown in fig. 11(a). The formation of beads was checked for three other rpm, say 1200, 1300, 1400 rpm and the diameter of microbeads was found as 135 µm as shown in fig. 11(b), 130 µm as shown in fig. 11(c), 127.5 µm as shown in fig. 11(d). From the graph it can be seen that as the rpm increased the bead diameter decreased.



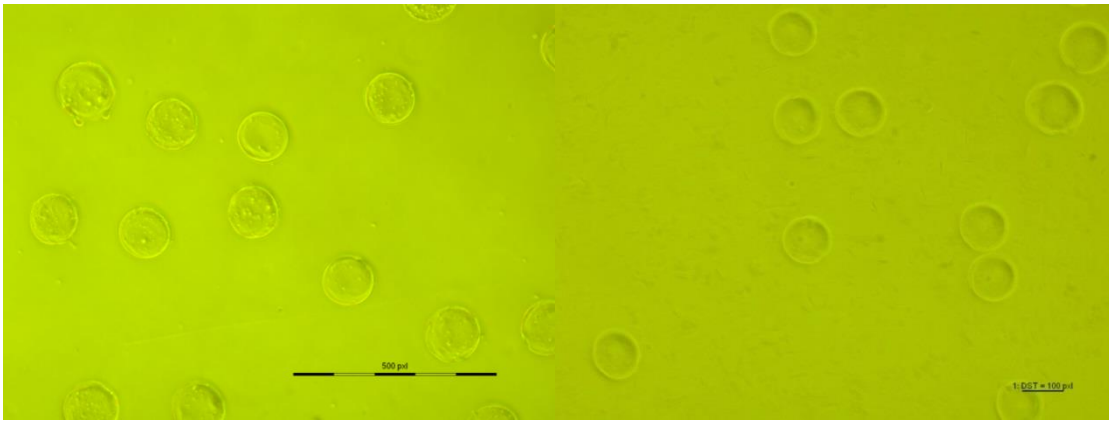
(a) **(b)**

(c) **(d)**

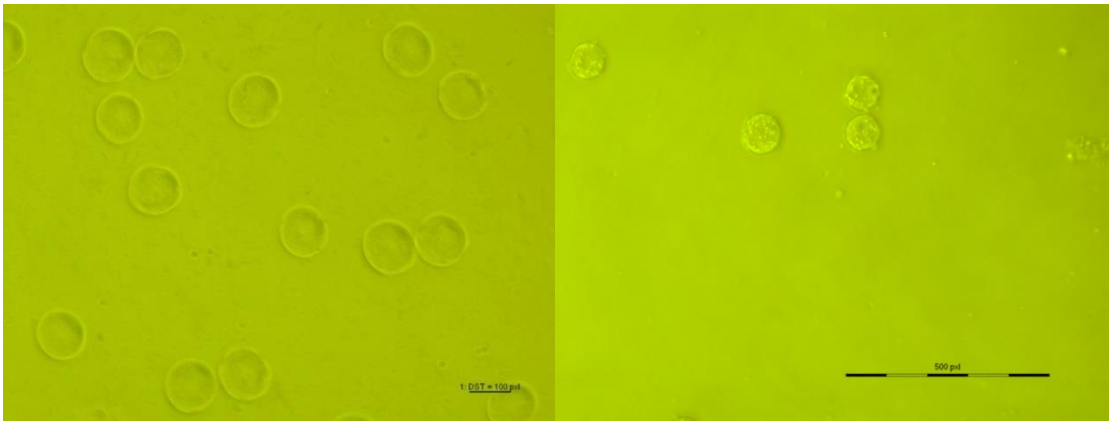
Fig. 11: Micro-alginate beads formed at (a) 1100 rpm having diameter 145µm (b) 1200 rpm having diameter 135 µm (c) 1300 rpm having diameter 130 µm (d) 1400 rpm having diameter 127.5 µm using 3% alginate and 26G syringe.

5.6 Micro-alginate beads from 3% alginate solution with 30G syringe needle (O.D (300 µm), I.D (148 µm))

There was no formation of beads at 1500 rpm. 1600 rpm was the least rpm at which beads formed. Diameter of beads was found to be 85 µm as shown in fig. 12(a). The formation of beads was checked for three other rpm, say 1700, 1800, 1900 rpm and the diameter of microbeads was found as 80 µm as shown in fig. 12(b), 75 µm as shown in fig. 12(c), 65 µm as shown in fig. 12(d). From the graph it can be seen that as the rpm increased the bead diameter decreased.



(a) **(b)**



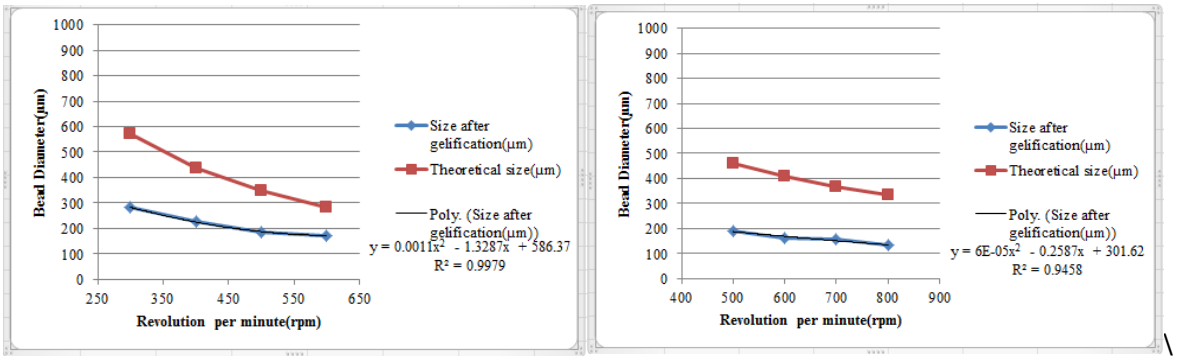
(c) **(d)**

Fig. 12: Micro-alginate beads formed at (a) 1600 rpm having diameter 85µm (b) 1700 rpm having diameter 80 µm (c) 1800 rpm having diameter 75 µm (d) 1900 rpm having diameter 65 µm using 3% alginate and 30G syringe.

5.7 Comparison of experimental bead diameter and theoretical bead diameter

Theoretical bead diameter was calculated from the two equation, one for surface tension and the other for centrifugal force and then equating those two equation. The theoretical diameter of Ca-alginate bead are systematically located above the experimental bead diameter. The reduction in diameter can be explained due to gelation process and accompanying shrinkage. Here as rpm is increased bead size decreases. This is due to inverse relationship of bead diameter and rpm which we get after equating surface tension force and centrifugal force.

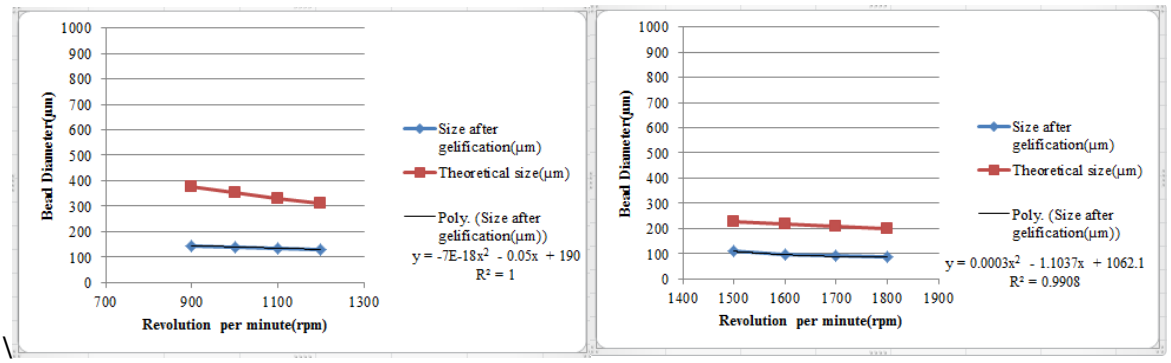
5.7.1 For 1% alginate solution



(a) **(b)**

Fig.13: Comparison of theoretical and experimental micro-alginate bead diameter for 1% alginate solution using (a) 26G needle(b) 30G needle.

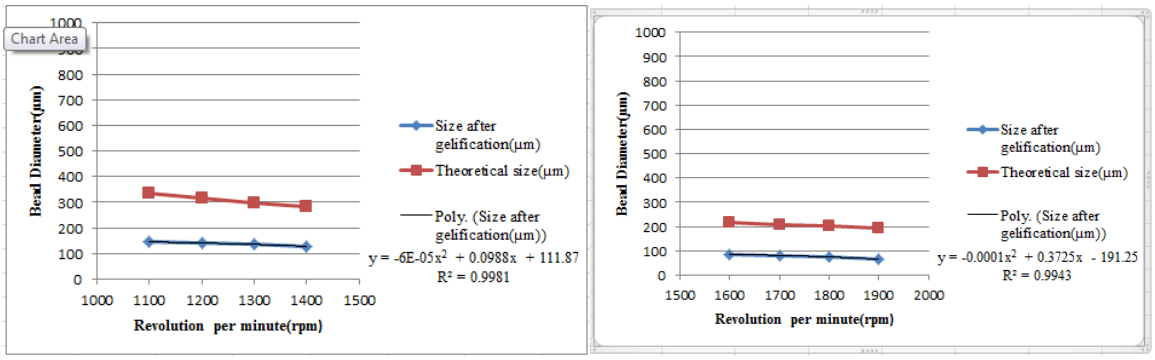
5.7.2 For 2% alginate solution



(a) **(b)**

Fig.14: Comparison of theoretical and experimental micro-alginate bead diameter for 2 % alginate solution using (a) 26G needle(b) 30G needle.

5.7.3 For 3% alginate solution

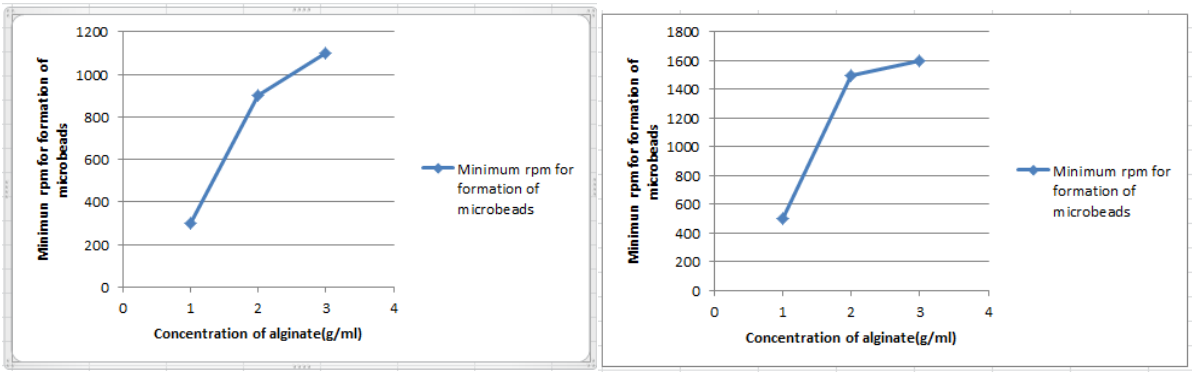


(a) **(b)**

Fig.15 Comparison of theoretical and experimental micro-alginate bead diameter for 3 % alginate solution using (a) 26G needle (b) 30G needle.

5.8 Variation of minimum rpm at which bead formed w.r.t its alginate concentration

As alginate concentration in increased, minimum rpm to form bead increases since there is direct relationship between rpm and alginate concentration which is derived from force balancing equation by equating centrifugal force and surface tension force.



(a) **(b)**

Fig. 16:Variation of minimum rpm at which beads formed with respect to its alginate concentration for (a) 26G needle and (b) 30G needle.

CHAPTER-6
CONCLUSION

6. CONCLUSION

This study added micro-bead fabrication capabilities to the recently introduced centrifugal multiphase microfluidic platform. By adjusting the spinning frequency and the nozzle geometry, the bead size produced can be as low as 65 µm. This size of the alginate microbeads is compatible with applications for therapeutic cell encapsulation. Compared to existing methods, this novel centrifugal scheme offers pulse-free und thus well reproducible droplet generation. The ability of the centrifugal encapsulation technology to even process highly viscous liquids within small diameter and thus high-resistance micro-nozzles under the impact of the centrifugally induced artificial gravity conditions has been shown for highly concentrated Na-alginate. Besides the capability to process high-viscous liquids, another advantage of the centrifugal microencapsulation technology is its conceptually simple and low-cost set-up.

CHAPTER-7
REFERENCES

7. REFERENCES:

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